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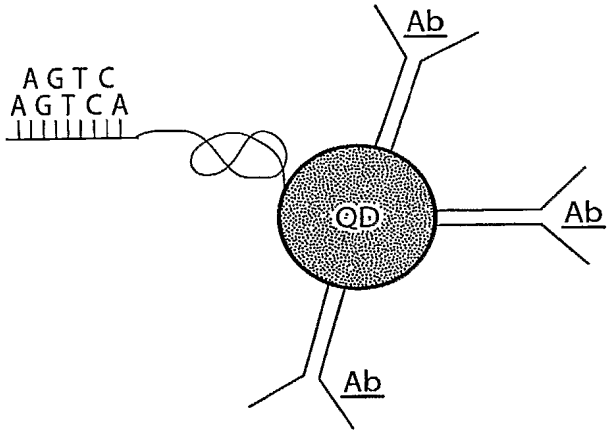
(54) Title: BIOHAZARD IDENTIFICATION BY FLUORESCENT IMMUNOASSAY AND SINGLE MOLECULE DETECTION

(57) Abstract: The invention provides methods and systems for real time detection and identification of biohazardous agents such as weaponized bacteria and the like.



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BIOHAZARD IDENTIFICATION BY FLUORESCENT IMMUNOASSAY AND SINGLE MOLECULE DETECTION

Field of the Invention

The present invention relates generally to methods and products for high throughput assays using a combination of single molecule detection, immunochemistry and microfluidics for the detection of rare agents.

Background of the Invention

In today's climate and heightened sensitivity to global terrorism, there exists a need for ways to detect weapons of bioterrorism such as chemicals and biologics. A detection system must be able to detect the biohazardous agent in real time so as to detect any danger or threat immediately and thereby stop its spread by introducing containment measures and identifying those exposed to the agent. The system must also be reliable as false positives can undermine its applicability and false negatives can be disastrous. Such systems would be used in a variety of places including public places such as airports, public transportation and office buildings.

These systems must be capable of sampling the environment and detecting biohazardous agents even if present in minute quantities. To be most versatile, the system should be able to detect a variety of biohazardous agents simultaneously.

Many of the systems currently available for detecting select biological agents are PCR based and thus require manipulation and synthesis steps which take time but may also introduce artifacts into the sample.

There is a need for a system that can rapidly and reliably detect a multitude of biohazardous agents in real time. Systems and methods derived for this purpose would also be useful in other applications including medical applications for the detection of rare factors or agents.

Summary of the Invention

The invention provides in its broadest sense a system that rapidly and reliably detects a multitude and variety of agents such as biohazardous agents. The system combines various technologies including large volume sampling (in some embodiments), microfluidics, single molecule detection capability, and immunoassay detection. It allows a wide variety of

biological and chemical agents to be detected simultaneously in order to, for example, identify a biohazardous situation quickly. The invention provides both "detect-to-protect" as well as "detect-to-treat" identification capability. The system does so in a cost-effective manner by recycling a subset of its most expensive components.

In one aspect, the invention provides a method for detecting a biohazardous agent comprising contacting a sample to a recycled first affinity partner conjugated to a first detectable solid support, contacting the sample to a recycled second affinity partner conjugated to a second detectable solid support, and determining binding of the first and second affinity partner to a biohazardous agent based on two presence of two color coincidence, wherein two color coincidence indicates the presence of the biohazardous agent. Contacting of the first and second affinity partners to the sample can occur simultaneously or sequentially.

In one embodiment, the recycled first affinity partner and/or the second affinity partner is present at a concentration of about 100 nM. The first and second affinity partners may be an antibody, an antibody fragment, or an aptamer, independent of each other. The first affinity partner and second affinity partner may bind to the same or a different epitope on the same biohazardous agent.

The first and second detectable solid support independently may be quantum dots. The quantum dots may be conjugated (or derivatized) to one or more oligomers, such as a single stranded nucleic acid oligomer. In one embodiment, the first detectable solid support and the second detectable solid support is fluorescently detectable.

In another embodiment, the sample is filtered. In yet another embodiment, the sample contains disrupted pathogens (e.g., as may occur following acoustic conditioning of the sample). In another embodiment, the sample has been concentrated by centrifugation. The volume is preferably volume condensed, although its entire volume may not be used in order to detect an agent. The sample may be derived from an air sample, a liquid sample, or a swab or swipe sample. In most embodiments, the sample is present in a solvent, such as but not limited to an aqueous solvent, such as a buffer.

In one embodiment, the first and second recycled affinity partners are recycled by application of a magnetic field.

In one embodiment, the biohazardous agent is a biological agent which may be a weaponized agent. The biological agent may be a bacterium, a bacterial spore, a virus, a

fungus, a parasite, a mycobacterium, or a mycoplasma. It may alternatively be a prion or a toxin.

In one embodiment, the method further comprises separation of first and second detectable solid supports that are bound to an agent from first and second supports that are not bound to an agent. In one embodiment, the separation is performed by chromatography.

In one embodiment, the two color coincidence is detected using a single molecule analysis system. In another embodiment, the single molecule analysis system comprises a parallel detection system. In yet another embodiment, the parallel detection system comprises a microlens system.

In another aspect, the invention provides a system for detecting a biohazardous agent comprising a sample collection device, a recycled first affinity partner conjugated to a first detectable solid support, a recycled second affinity partner conjugated to a second detectable solid support, a magnetic bead or particle derivatized with an third oligomer, a magnetic separation device, and a detection system. The first solid support is conjugated to a first oligomer and the second solid support is conjugated to a second oligomer. Each of the first and second oligomers have corresponding complimentary oligomers conjugated to a magnetic bead or particle such that each first and second support can be bound by a magnetic bead or particle via hybridization of complementary oligomers. The first and second oligomers may be identical or they may be different. Each first or second solid support may be conjugated to one or more oligomers.

In one embodiment, the sample collection device is an air sample collection device. In one method, the method further comprises a sample condensing device, which may also be the sample collection device. The system may comprise a microfluidic chip. In one embodiment, the detection system is a single molecule detection system, and it is preferably equipped with a fluorescence detector.

In still another aspect, the invention provides a method for detecting a biohazardous agent comprising contacting a sample to a recycled first affinity partner conjugated to a magnetic support, isolating the magnetic support, contacting the magnetic support with a second affinity partner that is detectable, and determining binding of the second affinity partner to the magnetic support, wherein binding of the second/secondary affinity partner indicates the presence of a biohazardous agent.

In one embodiment, the magnetic support is a magnetic bead or a magnetic particle. In another embodiment, the magnetic support is isolated by application of a magnetic field. In yet another embodiment, the second affinity partner is fluorescently detectable.

In another aspect, the invention provides a system for detecting a biohazardous agent comprising a sample collection device, a recycled first affinity partner conjugated to a magnetic support, a magnetic separation device, a second affinity partner that is detectable, and a detection system.

In one embodiment, the system further comprises a sample condensing device which may be the sample collection device. In another embodiment, the system comprises a microfluidic chip. In one embodiment, the magnetic support is a magnetic bead or a magnetic particle. In another embodiment, the detection system is a single molecule detection system. In yet another embodiment, the magnetic separation device is a magnetic field, for example as derived from a rare earth magnet.

Various of the afore-mentioned embodiments are equally applicable to this and other aspects of the invention and will not be reiterated. It is to be understood that such embodiments are relevant to the various aspects of the invention.

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments and to the accompanying drawings.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Brief Description of the Figures

FIG. 1 is a schematic diagram of one embodiment of the invention. (Legend: NS = antibody derivatized magnetic bead; E = enzyme; S = "dark" substrates that become fluorescent after enzyme catalyzed reaction.)

FIG. 2 is a schematic diagram showing the derivatization of a quantum dot with an affinity partner that is an antibody and a single stranded nucleic acid oligomer for recapture, recycling and regeneration of the binding assay. The quantum dot is considered a tri-functional reagent: it is itself directly detectable, its derivatized antibodies bind the agent to be detected, and the oligomer is used for recycling.

FIG. 3A shows the reaction scheme of second-order reaction kinetics for agent recognition.

FIG. 3B shows the progression of a typical sandwich immunoassay under select conditions. (Kinetic parameters: $k_A = 10^6\ M^{-1}s^{-1}$, and $k_D = 0.001\ s^{-1}$. Initial concentrations: 1 pM antibodies, and 15 aM agent. Solved by generalized 4th-order Runge-Kutta.)

FIG. 3C shows the kinetics of "antibody scrubbing" for agents. (Kinetic parameters: same as in FIG. 3B. Initial concentrations: 100 nM antibodies, and 15 aM agent.)

FIG. 4 is a schematic of a parallel GeneEngine™. High numerical aperture microlens objectives focus collimated excitation light into diffraction limited spots inside microfluidic channels, and separate beams of collected fluorescence emission are focused onto a single pinhole in the standard confocal configuration. The image plane at the microlenses (not the confocal spots) is re-imaged onto an array detector or a fiber-coupled array detector after spectral separation. Alternatively, the detector can be an imaging spectrophotometer.

The Figures are illustrative only and are not required for enablement of the invention disclosed herein.

Description of the Invention

In it broadest sense, the invention provides a method and system for detecting and identifying rare agents, as may be required in a number of settings including medical diagnosis applications. The method and system can also be applied to the detection of biowarfare agents. The method and system combine various methodologies including single molecule detection capability, immunochemistry, and microfluidics. The system contemplates the need for low cost, flexible biochemistry, and single molecule detection from large sample volumes. The end result is an inexpensive and rapid process for the identification of disparate hazardous agents.

The method and system provided by the invention has the capability to detect the presence of a biohazardous agent, regardless of its identity. This is most useful in instances where it is more important to discover a biohazardous threat and to limit exposure to it, for example by evacuation. The method and system also has the capability to identify the biohazardous agent (or agents) that is present. As will be discussed in greater detail below, due the ability of the method and system to multiplex (i.e., collect information for a plurality of agents simultaneously), it is possible to acquire information relating to all CDC category A and B agents in any given run. Regardless of which "mode" the system is running in, biohazardous agents are detected using agent-specific affinity partners that are specific for a given "epitope". In the "detect-to-protect" mode, every affinity partner may be labeled with the same detectable label (e.g., all may be derivatized to a green fluorescent quantum dot). In the "detect-to-treat" mode, each affinity partner may be labeled with a distinct detectable label (e.g., there will be one detectable label (or detectable label pair, as discussed in greater detail herein) per biohazardous agent).

In some embodiments, agent concentration may also be determined (in either of the above modes) where the amount of detectable label correlates with the number of bound agents. These readouts are then compared to a standard curve or to results from a simultaneous reference analysis using known concentrations of agents.

The invention can be applied to the detection and optionally identification and/or quantification of any agent, but most preferably rare agents which would otherwise be costly to detect. One example of such agents is biohazardous or biowarfare agents. These agents can be biological or chemical in nature. Biological biowarfare agents can be classified broadly as pathogens (including spores thereof) or toxins. As used herein, a pathogen (including a spore thereof) is an agent capable of entering a subject such as a human and infecting that subject. Examples of pathogens include infectious agents such bacteria, viruses, fungi, parasites, mycobacteria and the like. Prions may also be considered pathogens to the extent they are thought to be the transmitting agent for CJD and like diseases. As used herein, a toxin is a pathogen-derived agent that causes disease and often death in a subject without also causing an infection. It derives from pathogens and so may be harvested from such pathogens. Alternatively, it may be synthesized apart from pathogen sources. Biologicals may be weaponized (i.e., aerosolized) for maximum spread.

CDC Category A agents include Bacillus anthracis (otherwise known as anthrax), Clostridium botulinum and its toxin (causative agent for botulism), Yersinia pestis (causative

agent for the plague), variola major (causative agent for small pox), Francisella tularensis (causative agent for tularemia), and viral hemorrhagic fever causing agents such as filoviruses Ebola and Marburg and arenaviruses such as Lassa , Machupo and Junin.

CDC Category B agents include Brucellosis (Brucella species), epsilon toxin of Clostridium perfringens, food safety threats such as Salmonella species, E. coli and Shigella, Glanders (Burkholderia mallei), Melioidosis (Burkholderia pseudomallei), Psittacosis (Chlamydia psittaci), Q fever (Coxiella burnetii), ricin toxin (from Ricinus communis – castor beans), Staphylococcal enterotoxin B, Typhus fever (Rickettsia prowazekii), viral encephalitis (alphaviruses, e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis), and water safety threats such as e.g., Vibrio cholerae, Cryptosporidium parvum.

CDC Category C agents include emerging infectious diseases such as Nipah virus and hantavirus.

Other pathogens that can be detected using the methods of the invention include Gonorrhea, H. pylori, Staphylococcus spp., Streptococcus spp. such as Streptococcus pneumoniae, Syphilis; viruses such as SARS virus, Hepatitis virus, Herpes virus, HIV virus, West Nile virus, Influenza virus, poliovirus, rhinovirus; parasites such as Giardia, and Plasmodium malariae (malaria); and mycobacteria such as M. tuberculosis.

Examples of toxins include abrin, ricin and strychnine. Further examples of toxins include toxins produced by Corynebacterium diphtheriae (diphtheria), Bordetella pertussis (whooping cough), Vibrio cholerae (cholera), Bacillus anthracis (anthrax), Clostridium botulinum (botulism), Clostridium tetani (tetanus), and enterohemorrhagic Escherichia coli (bloody diarrhea and hemolytic uremic syndrome), Staphylococcus aureus alpha toxin, Shiga toxin (ST), cytotoxic necrotizing factor type 1 (CNF1), E. coli heat-stable toxin (ST), botulinum , tetanus neurotoxins, S. aureus toxic shock syndrome toxin (TSST), Aeromonas hydrophila aerolysin, Clostridium perfringens perfringolysin O, E. coli hemolysin, Listeria monocytogenes listeriolysin O, Streptococcus pneumoniae pneumolysin, Streptococcus pyogenes streptolysine O, Pseudomonas aeruginosa exotoxin A, E. coli DNF, E. coli LT, E.coli CLDT, E. coli EAST, Bacillus anthracis edema factor, Bordetella pertussis dermonecrotic toxin, Clostridium botulinum C2 toxin, C. botulinum C3 toxin, Clostridium difficile toxin A, and C. difficile toxin B.

Further examples of bacteria that can be used as biohazards include: Streptococcus spp., Staphylococcus spp., Pseudomonas spp., Clostridium difficile, Legionella spp.,

Pneumococcus spp., Haemophilus spp. (e.g., Haemophilus influenzae), Klebsiella spp., Enterobacter spp., Citrobacter spp., Neisseria spp. (e.g., N. meningitidis, N. gonorrhoeae), Shigella spp., Salmonella spp., Listeria spp. (e.g., L. monocytogenes), Pasteurella spp. (e.g., Pasteurella multocida), Streptobacillus spp., Spirillum spp., Treponema spp. (e.g., Treponema pallidum), Actinomyces spp. (e.g., Actinomyces israelli), Borrelia spp., Corynebacterium spp., Nocardia spp., Gardnerella spp. (e.g., Gardnerella vaginalis), Campylobacter spp., Spirochaeta spp., Proteus spp., Bacteriodes spp., H. pylori, and anthrax.

Further examples of viruses that can be used as biohazards include: HIV, Herpes simplex virus 1 and 2 (including encephalitis, neonatal and genital forms), human papilloma virus, cytomegalovirus, Epstein Barr virus, Hepatitis virus A, B and C, rotavirus, adenovirus, influenza A virus, respiratory syncytial virus, varicella-zoster virus, small pox, monkey pox and SARS virus.

Further examples of fungi that can be used as biohazards include: candidiasis, ringworm, histoplasmosis, blastomycosis, paracoccidioidomycosis, crytococcosis, aspergillosis, chromomycosis, mycetoma, pseudallescheriasis, and tinea versicolor.

Further examples of parasites that can be used as biohazards include both protozoa and nematodes such as amebiasis, Trypanosoma cruzi, Fascioliasis (e.g., Facioloa hepatica), Leishmaniasis, Plasmodium (e.g., P. falciparum, P. knowlesi, P. malariae,) Onchocerciasis, Paragonimiasis, Trypanosoma brucei, Pneumocystis (e.g., Pneumocystis carinii), Trichomonas vaginalis, Taenia, Hymenolepsis (e.g., Hymenolepsis nana), Echinococcus, Schistosomiasis (e.g., Schistosoma mansoni), neurocysticercosis, Necator americanus, and Trichuris trichuria.

Further examples of pathogens that can be used as biohazards include: Chlamydia, M. tuberculosis and M. leprosy, and Rickettsiae.

Examples of chemicals that can be detected include arsenic, arsine, benzene, blister agents/vesicants, blood agents, bromine, borombenzylcyanide, chlorine, choking/lung/pulmonary agents, cyanide, distilled mustard, fentanyls and other opioids, mercury, mustard gas, nerve agents, nitrogen mustard, organic solvents, paraquat, phosgene, phosphine, sarin, sesqui mustard, stibine, sulfur mustard, warfarin, tabun, and the like.

The foregoing lists of infections are not intended to be exhaustive but rather exemplary.

The invention uses a single molecule analysis system. Such a system is capable of analyzing single molecules either in a linear manner (i.e., starting at a point and then moving

progressively in one direction or another) and, as may be more appropriate in the present invention, in their totality. In certain embodiments in which detection is based predominately on the presence or absence of a signal, linear analysis may not be required. However, there are other embodiments embraced by the invention which would benefit from the ability to linearly analyze molecules (preferably polymers) in a sample. These include identification capabilities in which the identity of a pathogen will depend upon the number and positioning of affinity partners.

A single molecule detection system is capable of analyzing single molecules separate from other molecules. An example of such a single molecule detection system is the GeneEngine™ (U.S. Genomics, Inc., Woburn, MA). The Gene Engine™ system is described in PCT patent applications WO98/35012 and WO00/09757, published on August 13, 1998, and February 24, 2000, respectively, and in issued U.S. Patent 6,355,420 B1, issued March 12, 2002. This single molecule detection system routinely quantitates concentrations down to ~10 fM (femptomolar, 10^{-15} M) for dual-colored molecules using organic fluorophores and with two minute data collection times. Detection (i.e., determining presence or absence of an agent) is generally faster than quantitation (i.e., determining a concentration of agent), particularly for dual colored agents. For example, detection can occur in under one second if the agent is present in the pM range.

The GeneEngine™ platform can be adapted into a rapid automated biological identification system instrument. This can be accomplished by adding select functions to the platform, such as advanced microfluidics for agent concentration, and removing select components, such as the potentially unnecessary and expensive confocal optics.

The invention provides in a general aspect a method and system for detecting agents such as for example biohazardous agents and recycling the detection components repeatedly in order to reduce cost. The method is well suited to detecting rare agents such as those present at a concentration on the order to 500 pM or less (e.g., 10^5 fold lower). The invention contemplates manipulating the detection system in a number of ways depending on the rarity of the agent being detected. Any or all of these "enrichment" steps may be incorporated into the general method of the invention, depending on agent concentration. These enrichment steps include but are not limited to sample filtering, sample disruption (e.g., pathogen disruption), sample cleanup or separation (e.g., to remove affinity partners that are not bound to agent), and parallel detection. Each of these steps will be discussed in greater detail herein.

The system collects and delivers samples to a chamber such as a microfluidic chip that concentrates any molecule (including any biological agent) present in the sample and prepares it for detection. This requires sample collection, sample suspension in preferably liquid buffer (e.g., an aqueous buffer), and optionally crude or coarse filtering or separation.

Samples to be tested for the presence of biohazardous agents are generally taken from an indoor or outdoor environment. These include samples taken from air, liquids or solids in an indoor or outdoor environment. Air samples can be tested for the presence of normally airborne substances as well as aerosolized (or weaponized) chemicals or biologics that are not normally airborne. Air samples can be taken from a variety of places suspected of being biowarfare targets including public places such as airports, hotels, office buildings, government facilities, and public transportation vehicles such as buses, trains, airplanes, and the like.

Various air sampling devices are currently commercially available. As an example, BioAerosol Concentrator manufactures an air sampler having a size of 3.5 cubic inches. Other companies that manufacture air sampling devices include International pbi S.pA. (making a device that aspirates 1 cubic meter is 10 minutes, or 1 cubic meter in 6 minutes, or 1 cubic meter in 3 minutes), Meso Systems, Sceptor Industries, Inc., and Anderson. Moreover, techniques for air sampling are described in J.P. Lodge, Jr. Methods of Air Sampling and Analysis, Third Edition, Lewis Publishers, Inc. (December 31, 1988) ISBN 0873711416.

Liquid samples can be taken from public water supplies, water reservoirs, lakes, rivers, wells, springs, and commercially available beverages. If necessary, concentration of liquid samples can be done by centrifugation, evaporation, lyophilization, and the like.

Solids such as food (including baby food and formula), money (including paper and coin currencies), public transportation tokens, books, and the like can also be sampled via swipe, wipe or swab testing and placing the swipe, wipe or swab in a liquid for dissolution of any agents attached thereto. Again, based on the size of the swipe or swab and the volume of the corresponding liquid it must be placed in for agent dissolution, it may be necessary to concentrate such liquid sample prior to further manipulation.

Air, liquids and solids that will come into contact with the greatest number of people are most likely to be targets of biohazardous agent release.

Sampling can occur continuously, although this may not be necessary in every application. For example, in an airport setting, it may only be necessary to harvest randomly

a sample near or around select baggage. In other instances, it may be necessary to continually monitor (and thus sample the environment). These instances may occur in "heightened alert" states.

An exemplary air sampling protocol would collect material from 1000 L of air and resuspend it in no more than 1 mL of buffered water. The buffering is required to ensure integrity of any agents contained therein and to prevent denaturation of any epitopes recognized by the primary and secondary affinity partners. The simplest buffers will at least maintain a pH in the range of about 7, or a pH that is compatible with the biological being detected, and may optionally contain various analytes such as cations and anions. The buffer may also contain preservatives in some instances. The analyses provided herein assume in some aspects that this volume of air (i.e., 1000 L) will contain, if anything, 100 pathogens or 50 pg of toxin per L and that such amounts will be collected into the 1mL aqueous buffer at 90% efficiency.

At 90% collection efficiency, it is expected that the starting agent concentration can be approximately 15 aM = 1.5 x 10^{-17} M for biological agents such as pathogens, and approximately 50 pM = 5 x 10^{-11} M or higher for toxins. Toxin concentrations of 50 pM will be detected by the GeneEngine™ which has current detection limit of ~10 fM). Pathogen levels that are significantly lower may require further concentration prior to analysis. However, such a calculation assumes that only one antigen is presented per pathogen. In fact, each pathogen is expected to present many antigens each of which can be recognized by the primary and secondary affinity partners of the invention. Thus, the effective local concentration of antigens may actually approach the GeneEngine™ detection limit without further manipulation. Therefore, further concentration might be unnecessary on a sensitivity basis alone.

In other embodiments, however, it may be necessary to concentrate a sample prior to analysis. The invention proposes two pre-analysis steps for increasing the effective concentration of agent to be detected: pathogen disruption and centrifugation. One, both or neither of these steps may be necessary depending on the agent concentration in the sample or the efficacy of either step.

In order to increase "epitope" density of the sample, it may be necessary to disrupt pathogen cell walls, cell membranes or viral envelopes, thereby releasing a plurality of epitopes, both intracellular and extracellular. This serves to "enrich" for agents (or agent markers such as epitopes) to be bound by the affinity partners, and ultimately increases the

number of affinity partners having an agent bound thereto. It is to be understood that the affinity partners of the invention are not limited to antibodies (as described below). However, for the sake of convenience, the disclosure refers to "epitopes" generically to mean that agent (or agent marker) that is recognized by the affinity partner, regardless of whether that affinity partner is an antibody or an aptamer, or the like.

Disruption can be accomplished by any number of means including mechanical, electrical, osmotic, pressure, and the like. In one embodiment, the sample is exposed to an acoustic conditioning method.

As an example, microorganisms can be disrupted using a non-contact, reagent-less focused acoustic technology, developed at Covaris Inc., Woburn, MA, and described in U.S. Patent No. 6,719,449, issued April 13, 2004. This procedure enables higher recoveries and better reproducibility than conventional, physical contact systems such as liquid nitrogen grinding, bead beating, sonicators (low frequency, unfocused, standing waves) and polytron-type homogenizers. In addition, it does not require special reagents.

The only evidence of sample alteration as a function of the acoustic conditioning process is the shearing of genomic DNA. At high cell densities, sample viscosity increases dramatically on release of intact genomic DNA during cell lysis. Typically small amounts of DNase can negate this effect, but not instantaneously. The acoustic conditioning process has the side benefit of reducing or eliminating altogether this problem. By adjusting the control parameters (power, frequency and acoustic field topology) a desired level of shearing can be obtained.

The standard acoustic conditioning instruments are designed for 0.1 to 10.0 mL processing volumes. The volume of the focal zone of the process scales with the frequency. Transducers and driver electronics are scaled to match sample and fluid volumes. The power requirement is directly proportional to the mass of the sample to be processed; therefore the power required for the small sample sizes will be low. Frequencies in about the 1.1 MHz frequency are contemplated.

Agent (particularly pathogen) concentrations may also or alternatively be increased by centrifugation. Centrifugation is expected to increase agent concentration on average by about 10-fold. *E. coli* suspensions can be spun down in under 30 seconds using inexpensive benchtop centrifuges (e.g., Eppendorf), but many of the CDC Category A and B pathogens are smaller and therefore sediment more slowly. For example, the Marburg virus is the smallest (~20-fold smaller logmean size than *E. coli*) and would take around ten minutes to

pelletize. However, solid pellets are preferably to be avoided because their resuspension would require an extra step. Instead, it is preferable to form a loose pellet at the bottom and extract a small volume directly therefrom.

The sample may be filtered to remove large insoluble particulates that are irrelevant to and may obscure the assay. Filtration (or essentially physical separation of debris from the sample) can be accomplished in a number of ways including but not limited to mild centrifugation (to be distinguished from the centrifugation used to concentrate biologicals in the sample, as discussed herein). One of ordinary skill in the art will be able to determine the necessary g forces to sediment large particles (e.g., those having an effective diameter or length of approximately 1 mm or more) versus those required to sediment smaller particles (e.g., those having an effective diameter or length of less than 250 microns, less than 100 microns, less than 20 microns, and equal to or less than 5 microns, the latter of which are most likely to enter the lungs).

The analysis or binding step of the method involves contacting the sample (and the agents or fragments contained therein) with agent-specific affinity partners. The affinity partners of the invention are not limited in nature provided they recognize and bind to their respective targets with sufficient specificity. Binding with sufficient specificity means that they do not bind to other substances at all or at least not at an appreciable rate. For example, a suitable affinity partner is one that demonstrates non-specific binding with an affinity that is at least 5-fold less, at least 10-fold less, at least 100-fold less, or at least 1000-fold less than the affinity for its target.

The art is familiar with various types of affinity partners including binding peptides such as antibodies and antigen-binding fragments thereof. Synthetic or naturally occurring peptide libraries can also be screened for peptides that bind to biological or chemical agents of interest with the desired affinity.

Antibodies are known in the art. Polyclonal or monoclonal antibodies may be used. The antibodies may derive from any naturally occurring source (e.g., human, mouse, rat, avian species, and the like) and they may be chimeric. Antibody classes of IgA, IgG, IgM, IgD, IgE are all contemplated by the invention. Antibody fragments most useful in the invention are those that are antigen-binding (e.g., the antigen binding domain of the parent antibody having the same specificity as the parent antibody). Antibody fragments include but are not limited to Fab, $F(ab)_2$, Fv, single chain variable fragments (scFv), and single chain antibodies.

Examples of antibodies or antibody fragments that are specific to biological and chemical agents are available from CHEMICON International (Temecula, CA), and BIODESIGN International (Saco, Maine), and others have been described by Berry et al. J. Virol. Methods, 2004, 120(1):87-96 (monoclonal antibodies to SARS), Traggiai et al. Nat Med. 2004, 10(8):871-5 (human monoclonal antibodies to SARS), Che et al. Di Yi Jun Yi Da Xue Xue Bao. 2003, 23(7):640-2 (mouse monoclonal antibodies to SARS), Shin et al. J Parasitol. 2004, 90(1):161-6 (monoclonal antibodies to Toxoplasma gondii), and Bregenholt et al. Expert Opin Biol Ther 2004, 4(3):387-96 (pathogen-specific recombinant human polyclonal antibodies). For use in governmental equipment or for government mandated or sponsored surveillance programs, affinity partners such as antibodies or antigen binding fragments thereof are also available from the Critical Reagents Program of the U.S. Government, which maintains critical reagents for such uses and sets standards for the reagents to be used in these applications.

Affinity partners that are nucleic acid in nature (i.e., aptamers) are also known in the art and have been used routinely for analyte detection. Aptamers can be made that are specific for virtually any analyte including the biological and chemical agents of the invention. Aptamer technology is described in greater detail in US Patent Nos. 5,270,163; 5,475,096; 5,567,588; 5,595,877; 5,637,459; 5,660,985; 5,670,637; 5,683,867; 5,696,249; 5,705,337; 5,707,796; 5,789,157; 6,011,020; and 6,261,774. Examples of aptamers specific for biological or chemical agents include but are not limited to those described by Jeon et al. J Biol Chem. 2004 Sep 8; Sayer et al. J Biol Chem. 2004, 279(13):13102-9 (prion specific aptamer), and Bruno et al. Biosens Bioelectron. 1999, 14(5):457-64 (anthrax spore specific aptamer).

In still other embodiments, the affinity partners may be riboreporters, as described in U.S. Patents 4,987,071; 5,093,246; 5,589,332; 5,591,610; 5,741,679; 5,834,186; 6,025,167; and 6,180,389.

It is to be understood that the nature of the primary affinity partner is independent of the nature of the secondary affinity partner. Thus, for example, the primary and secondary affinity partners may both be antibodies or antibody fragments, or they may both be aptamers. Alternatively, one may be an antibody or an antibody fragment and the other may be an aptamer.

For the sake of convenience, this disclosure often refers to the affinity partners as antibodies. It is to be understood however that the affinity partners can be any number of

substances provided they can bind specifically to the agent to be detected. Thus, for example, the affinity partner can be an aptamer or a ribozyme also.

Depending on the embodiment, the primary affinity partners may bind specifically to a moiety that is present on more than one biological agent but is not present on other substances. Thus, it is able to capture biological agents but will not capture other substances non-specifically. In this way, it is possible to capture a plurality of different biological agents with only one type of affinity partner. This reduces the cost and time required for derivatizing solid supports such as magnetic particles or beads or quantum dots with a particular affinity partner for each biological to be captured. Alternatively, however, the method may employ one primary affinity partner for each biological or chemical agent to be captured. Depending on the embodiment, the same is true for secondary affinity partners.

Primary and secondary affinity partners may bind to the same or to different epitopes on a biological agent. If they bind to the same epitope, then that epitope must be expressed at sufficiently high levels on the agent to preclude detrimental competition between the primary and secondary affinity partners.

The invention contemplates using the least amount of affinity partner per reaction in order to reduce costs. Antibody and quantum dot or magnetic bead/particle concentration will range from about 20nM to 500 nM. Since there are approximately 5 antibodies per quantum dot, the concentration of antibodies will be 5-fold higher than that of the quantum dots (e.g., 100 nM of antibody will correspond to 20 nM of dots, beads or particles).

The invention contemplates the ability to detect a plurality of agents in any given run. For example, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or more agents can be screened for simultaneously.

Agents can be distinguished based on different detectable readouts such as different fluorescence signals. In one embodiment, each of the agents can be uniquely identified based on a unique detectable label. The detectable label may be directly or indirectly conjugated to the affinity partner or to the solid support to which the affinity partner is itself affixed. An example of a directly detectable label is a quantum dot. Fluorescence of a quantum dot is governed by its diameter; therefore it will be possible to make a series of quantum dots each with its own distinct agent-associated signal. As will be discussed in greater detail herein, it may be preferable in some embodiments to detect an agent via the coincident presence of two colors, as may be possible if two quantum dots, each with a distinct emission spectrum, have

the same binding specificity. In this way, one agent can be bound to two quantum dots and will be associated with the coincident emission from both dots.

An example of an indirectly detectable label is an enzyme which is detected via its ability to cleave or convert a substrate into a detectable product. A specific enzyme (and thus detectable product) can be assigned to each agent to be detected. The indirectly detectable label may be conjugated to a primary and/or a secondary affinity partner, or a solid support. As an example, a secondary affinity partner specific for anthrax spores may "read-out" as a green fluorescence (due to the production of a "green" product) while the secondary affinity partner specific for smallpox may "read-out" as a red fluorescence (due to the production of a "red" product). In yet another permutation of these systems, it is possible that the primary affinity partner (and its corresponding solid support) is permanently located at a known region on the chip, some or all secondary affinity partners are conjugated to the same enzyme, but to each defined discrete location on the chip is applied a different substrate that converts into a different colored product.

In other embodiments, signals from different agents can be differentiated by location on a chip (or other solid support amenable to microfluidic manipulation), whereby for example the affinity partners that are specific for a single agent are permanently located via the solid support to which they are affixed. In this set up, presence or absence of a signal in a defined region on the chip indicates presence or absence of the particular agent.

An exemplary reaction strategy is a fluorescence sandwich immunoassay: two labeled affinity partners (e.g., antibodies) bind agents simultaneously. As discussed in greater detail herein, affinity partners may be directly or indirectly fluorescent. The antibodies must be capable of binding the agent at the same time, thereby enabling two signals to emit from each doubly bound agent. In other words, binding of one affinity partner should not preclude binding of the other affinity partner. Differentially labeled antibodies can bind to different "epitopes" or the same "epitope", provided that in the latter case there are more epitopes on the agent than there are antibodies capable of binding them (i.e., the first and second antibodies will not outcompete each other even if they bind to the same epitope). It is to be understood however that the first and second (or primary and secondary) antibodies may be contacted with a sample (or agent) at the same time or sequentially.

It may be possible in some embodiments to detect confidently the presence of an agent using single color detection. However, in other embodiments, the use of two color coincidence increases the confidence level of the detection system. This may be particularly

important where false positives will create much inconvenience. As used herein "two color coincidence" is the use of two signals bound to an agent to indicate the presence of the agent.

One embodiment of the invention is schematically outlined in FIG. 1 which shows a three step reaction. The first step is separation, concentration, and washing of the biological or chemical agents. The second step completes the sandwich immunoassay by binding recyclable (and recycled) derivatized secondary affinity partners (such as secondary antibodies) to alternate assessable epitopes. Having concentrated the biological or chemical agents at least 100×, the second reaction can be expected to proceed more quickly than the first. Nevertheless, the antibodies must be at a very high concentration for the same reasons of reaction speed and progression, and consequently must be reused. To accomplish this, the secondary antibody buffer is evacuated from the mixing chamber and placed in a storage chamber, leaving the magnetic beads in place. The mixing chamber must be thoroughly washed to remove unbound secondary antibodies before the third reaction to avoid false positives.

The affinity partners of the invention are preferably affixed to a solid support, and more preferably to a solid support that is itself not permanently fixed to another support. Even more preferably, the solid support enables the recyclization of the affinity partner. Thus, for example, the affinity partner may be conjugated to (or derivatized to) a magnetic bead or particle which can be harvested and reused between detection and binding steps. Such particles or beads are commercially available from sources such as Bioclone Inc. (San Diego, CA), Dynal Biotech, supplier of Dynabeads (Oslo, Norway), BD BioSciences, supplier of IMags, and BioMags. These commercial vendors are able to derivatize particles or beads with affinity partners of choice. Alternatively, methods for derivatizing particles or beads with affinity partners are provided by these manufacturers.

In some embodiments, particularly those using magnetic beads as a solid support, a second affinity partner is applied to the particles or beads that have already been exposed to the sample. The second affinity partner may bind to a different epitope on the captured agents than does the first affinity partner. If the first and second affinity partners are antibodies, then this is reminiscent of a conventional sandwich immunoassay. The second affinity partner can be derivatized in many ways. As an example, the second affinity partner can be derivatized with an enzyme that reacts with a substrate to yield detectable products. The enzyme-substrate pair can be selected so that the substrate is not detectable at a given wavelength but products thereof are detectable at that same wavelength. Use of enzyme-substrate labeling

systems allows for an amplified detection system since one enzyme will be able to convert a multitude of substrates into detectable products. Other amplifiable systems are also suitable for use in this system.

Secondary affinity partner labeling systems that are capable of amplification are better suited to single molecule detection since they facilitate stronger signals from individual agents. One example of such an amplifiable labeling system is a fluorescence-based system in which each fluorophore can undergo many photocycles resulting in significant signal amplification. In addition, the use of confocal optics greatly minimizes background signals. And modern photodetectors having quantum efficiencies approaching one further facilitate single molecule detection since less of each signal is lost to photodetector inefficiency. A wide variety of fluorescence approaches are available to minimize false positives, including correlation analysis between multiple colors (e.g., when using a two color fluorophore), spectral deconvolution, fluorescence decay lifetime, fluorescence correlation spectroscopy, and more.

As another example of a solid support, the affinity partner may be conjugated to (or derivatized to) a solid support that is a fluorescent particle. An example of a suitable fluorescent particle is a quantum dot. Quantum dots are fluorescent nanocrystals made of a core material and a shell material. The core material is comprised of a semiconductor material such as cadmium sulfide (CdS), cadmium selenide (CdSe) or cadmium telluride (CdTe). The core material essentially controls the emission wavelength of the dot: CdS emits in the UV-blue, CdSe emits in the visible range, and CdTe emits in the far red to near-infrared. The cores can be as small as 10^{-9} diameter spheres, but they may also be shaped as rods, pyramids, boomerangs, tetrapods, and the like. The size of the dot therefore correlates with its emission spectrum, with larger dots emitting longer wavelengths (e.g., a 6 nm diameter dot emitting in the 655 nm range) than smaller dots (e.g., a dot that is less than 3 nm diameter emitting in the 525 nm range). Quantum dots also comprise a shell made of a non-emissive transparent material that is compatible with the core material. The shell material helps to smooth out imperfections in the surface of the core material.

Quantum dots are particularly suited to some embodiments of the invention. In comparison to typical organic fluorophores, quantum dots have a 100- to 1000-fold increase in brightness (estimated as the quantum yield times the extinction coefficient), a single broad absorbance band for all colors that is blue-shifted >100 nm from emission, narrow emission peaks, relative insensitivity to environment, and high photostability. Each of these

characteristics are useful when detecting rare agents, and particularly when information from a plurality of agents is desired in a given run (i.e., when multiplexing is desired).

Generally, fluorescence signal to noise (S/N) ratios are enhanced in proportion to the relative brightness of the fluorophores. Quantum dots are substantially brighter than organic fluorophores. Greater than ten-fold S/N ratio improvement has been observed using quantum dots and unoptimized single molecule detection systems (e.g., GeneEngine™). By optimizing the excitation source and light filtration, at least another 10-fold improvement is expected. The broad absorbance peak that is well separated from the emission peaks and the ability to use a single excitation source combine to reduce detection costs even more.

Multiplexed detection and other spectral analyses are facilitated by the narrow emission peaks of quantum dots, the single wavelength excitation capabilities, and their environmental insensitivity. Multiplexed detection is important for parallel detection and/or identification.

In order to reduce errors further, optionally the method can comprise taking two simultaneous measurements: one at the emission optimum of the quantum dot and the other outside the optimum. Quantum dots will exhibit a large difference in fluorescence between these two measurements. Any naturally occurring source of fluorescence, many of which can be present in air samples, will exhibit comparable signals in wavelengths or wavelength ranges. Quantum dots are also durable against the environment and photodamage, making them a suitable candidate as a recycled reagent. QDs are comparatively small (<20 Å including the hydration shell) and only require approximately five or fewer antibody-sized macromolecules for broad surface coverage. (See for example Qdot™ Protein A Conjugates User Manual", Cat. # 1020-1, 1022-1, Quantum Dot Corporation).

Solid supports such as the magnetic beads or particles or the quantum dots can be directly or indirectly derivatized with the various functionalities. Direct derivatization is preferred if possible. In the case of quantum dots, direct derivatization is further preferred because it avoids increasing the size of the dot (and thereby requiring more antibody bound thereto). Indirect derivatization involves the use of for example streptavidin coated magnetic beads or particles or quantum dots in conjunction with biotinylated affinity partners and oligomers. With respect to quantum dots, the streptavidin coat will double the particle diameter adding marginally to the reaction cost by consuming only slightly more antibodies. Derivatization of quantum dots has been described in U.S. Provisional Application Serial No.

60/520,927, filed November 17, 2003, and entitled "Single Center Quantum Dots for Fluorescent Tagging", the entire contents of which are incorporated herein.

As with the antibodies, the oligomer may be directly or indirectly derivatized to the quantum dot. In one embodiment, it may be attached to the quantum dot via a flexible linker. These linkers can be any of a variety of molecules, preferably non-active, such as straight or even branched carbon chains of carbon, saturated or unsaturated, phospholipids, and the like, whether naturally occurring or synthetic. Additional linkers include alkyl and alkenyl carbonates, carbamates, and carbamides. The oligomer may also be derivatized to the quantum dot via streptavidin-biotin.

Long reaction times and high costs are obstacles to be overcome by the invention, particular as they are more prevalent in rare agent detection. Immunoassay based approaches are generally driven by second-order chemical kinetics. However, in the case of low abundance agents, the relative antibody (Ab) concentrations ([Ab]) required for detection are typically very high causing pseudo first-order reaction kinetics with respect to [agent]:

$$\frac{d}{dt}[\text{agent}] = -k_A[\text{Ab}][\text{agent}]$$
$$\approx -k_A'[\text{agent}]$$
$$\frac{[\text{agent}](t)}{[\text{agent}](t=0)} \approx \exp(-k_A' t)$$

Consequently the percent reaction completion time is independent of [agent] and can only be affected by changes in [Ab] or the second-order rate constant, k_A. (The reverse dissociation reaction is much slower at high [Ab], and therefore contributes negligibly.) From the Smoluchowski equation (von Smoluchowski, M., 1917, Versuch einer mathematischen Theorie der Koagulationskinetik kolloider Lösungen. *Z. physik. chemie.* 92:129-168),

$$k_A = 4\pi a D f,$$

(where a is the center-to-center distance at closest approach, D is the relative diffusion coefficient, and f is the sterical factor), the large radius of microspheres does not hinder reaction progression so long as the Ab surface density is sufficient to maintain a high sterical factor (related to the fraction of active surface area). In essence, the slower diffusion of a larger bead is more than compensated for by its greater active surface area:

$$k_A \propto (r_{Ag} + r_{bead})\left(\frac{1}{r_{Ag}} + \frac{1}{r_{bead}}\right)$$

$$\propto \frac{r_{bead}}{r_{Ag}}, \text{ when } r_{Ag} \ll r_{bead},$$

where r_{Ag} and r_{bead} are the antigen (Ag) and derivatized bead radii respectively. To achieve solid support - Ag binding rates comparable to Ab-Ag reactions, the Smoluchowski equation requires Ab solid support surface densities of ~10^4 to 10^5 μm^{-2}. Since derivatizing solid supports such as beads requires high amounts of antibodies (and is therefore costly), the invention limits the use of antibody reagents by using single molecule detection capability (as provided by for example GeneEngine™) and by capturing agents with recyclable Ab-derivatized solid supports.

FIG. 3 demonstrates the scarcity of reactants and importance of efficiently using reagents for the sandwich immunoassay. Consider the first case shown in FIG 3B. It is assumed that pathogens are collected into a 1 mL reaction buffer from 100 L of air at 100 parts per liter, for a final concentration of 15 attomolar (aM = 10^{-18} M). Assuming a hypothetical cost constraint of 8¢ per reaction, the maximum Ab concentration afforded is ~1 pM. The equilibrium concentration of identifiable targets is vanishingly small: approximately six dual-labeled pathogens per L, or none per mL on average. Thus, not only is the agent concentration small, but very little antibody is afforded per reaction. Reaction performance would only be marginally improved by antibody engineering. Instead, the invention proposes increasing the amount of antibody used per reaction and recycling such antibody reagents in order to reduce costs over a plurality of reactions.

FIG. 3C demonstrates that over 90% of the pathogens initially present can be dual fluorescently tagged within 30 s at very high antibody concentrations (e.g., 100 nM). Reaction progress was estimated using association rates for free antibodies in clean buffer. Actual reaction progress might be slightly slower caused by miscellaneous debris in the sample. The rate of agent capture must also be balanced against reagent recycling. For example, in a 1 mL reaction with 100 nM antibodies, the antibody reagents must be reused 10^5 times, or equivalently must stay in service for as long as ~4 months. Lowering the antibody concentration eases the demand on durability, but lengthens the time needed for agent tagging. Remarkably, 1 µl of antibody would only need to last a few hours by minimizing reaction volumes.

Reagent concentration must be balanced against both the cost per reaction and the limit of detection. FIG. 3C demonstrates the principle that all CDC Category A and B agents can be successfully modified for detection by a general approach. However the high probe concentrations required pose a challenge for observing the bound, tagged complexes among the great excess of free tags.

Initial toxin concentrations are estimated at 500 pM, yielding a ~100:1 free tag to complexed tag-toxin ratio after a 30 second reaction ([toxin] << [antibody], therefore by pseudo-first order approximation the reaction progression in FIG. 3C is independent of [toxin]). The limit of detection is approximately 1000:1 tag-to-toxin ratio. Therefore, toxins can be detected easily as their prevalence is above the limit of detection.

Some pathogen concentrations on the other hand are 10^5-fold lower than toxin concentration (even taking into account a 10-fold concentration by centrifugation or another upstream approach) for a final 10^7:1 tag to pathogen ratio. This exceeds the detection limit. Acoustically conditioning the collection sample prior to the binding step serves to increase the effective pathogen concentration by disrupting and fragmenting cell walls and membranes and viral envelopes. This should result in an average of 100 intact fragments per pathogen to increase the free tag to pathogen ratio up to 10^5:1. To reach the detectable 10^3:1 ratio, other steps may be incorporated such as high throughput size-separating chromatography.

If necessary, reaction clean-up can be performed using various techniques including but not limited to field flow fractionation (FFF) (Liu, M-K., & Giddings, J. C. (1993) Separation and Measurement of Diffusion Coefficients of Linear and Circular DNAs by Flow Field-Flow Fractionation. *Macromolecules* 26(14):3576-3588; Wyatt, P. J. (1998) Submicrometer Particle Sizing by Multiangle light scattering following fractionation. *Journal of Colloid and Interface Science* 197: 9-20), or hydrodynamic chromatography (HDC) (Chmela et al. (2002) A Chip System for Size Separation of Macromolecules and particles by Hydrodynamic Chromatography. *Anal. Chem.* 74(14): 3470-3475). FFF represents a series of high-resolution chromatographic techniques used to separate particles based on size. Among the unique attributes of FFF are its extremely wide dynamic range (1 nm to 100 μm), short preparation time (1-10 min), and absence of an immobile phase. Briefly, one dimension of an FFF chamber is small enough to ensure laminar flow. Hydrodynamic flow drives mixtures through the chamber while a perpendicular force on the solutes is applied to displace the solutes towards the decelerated flow at the chamber boundary. A narrow gap ensures a strong velocity gradient at the boundary, where smaller, faster-diffusing particles escape the flow's

slowest regions more readily than large particles. Consequently, small particles emerge first at the outlet. In contrast, HDC works in reverse with the same laminar flow through a shallow and wide channel but without a tangential force. In this case, the displacement of the smaller particles is delayed by their greater ability to diffuse into slow regions of the flow.

FFF devices have used electricity, sedimentation, and tangential flow to produce the force. Flow FFF (FlFFF) has a wide dynamic range, flexibility, and uniform response over different solute types. FlFFF units have a porous boundary that produces tangential flow.

The separation and injection components of HDC are compatible with standard lithography, enabling all components of agent binding and detection to be implemented on one chip. Moreover, HDC performance surpasses requirements for pathogen fragment separation from free tags and offers an orthogonal size-based approach to confirm agent detection that may even confirm identification. Prior separation equivalent to a ~1000-fold clean-up between 26 and 44 nm spherical beads was reported using HDC over three minutes. (Chmela et al. (2002) A Chip System for Size Separation of Macromolecules and particles by Hydrodynamic Chromatography. *Anal. Chem.* 74(14): 3470-3475). Although unnecessary, this performance level is probably sufficient even for some level of clean-up of the toxin reactions (the ratios of diffusion coefficients are ~1.7 *vs.* ~1.4 for the beads in the study *vs.* our reactants respectively). (Approximating tags as spheres of radius r, and toxin-tags as ellipses with axes ~r and ~$2r$, the free tag diffusion coefficients are only ~1.4 times higher. Berg, H. C. (1993) Random Walks in Biology, Princeton University Press, Princeton, NJ, pp. 56-57.)

Clean-up of the much larger pathogen fragments should more than exceed 1000×, although some of this advantage is lost to separation and dilution of the differently sized fragments. In some embodiments, pathogen disruption may be eliminated entirely and a clean-up step such as chromatography alone is used in order to concentrate agent.

Rapid detection and/or identification of rare agents by fluorescence single molecule detection can be further aided by scaling up throughput. The GeneEngine™ currently scans approximately 1 nL of fluid in two minutes. In some embodiments, twenty times that amount should be scanned to overcome the high free tag to target ratio. One way of accomplishing this is to by parallelizing detection. This can be done for example with either on-chip microlenses or holographically generated spot arrays with array detection for greater throughput and substantial system cost reduction. The different components in the proposed system are described below.

The high quality microscope objectives used in confocal microscopes, such as in the GeneEngine™, are both expensive and bulky, and essentially limited to a single light collecting element. All parallelization must occur within the area imaged from that element. One approach to parallelization is replacement of the objective with a microlens array, as shown in Figure 4. An objective based approach is discussed as an alternative below (i.e., confocal spot array).

Microlens fabrication is a mature technology and accordingly the corresponding properties are understood. Among the advantages of microlenses are adequate light collection efficiency and optical resolution when used with ultrabright fluorophores (demonstrated up to 0.64 numerical aperture) (Jeong, K-H & Lee, L. P. (2002) A New Method of Increasing Numerical Aperture of Microlens for Biophotonic MEMS. *The 2nd Annual International IEEE-EMBS Special Topic Conference on Microtechnologies in Medicine and Biology*, Madison, WI, pp. 380-383), fixed focus with respect to the fluidic channel, very low unit costs in large production, and high surface densities on chips for maximum parallelization.

Microlenses can work in single molecule detection applications exploiting ultrabright fluorophores such as quantum dots. The light gathering power of even the highest numerical aperture microlens is significantly lower than for a high quality objective (~5× lower; a 0.64 *vs.* 1.4 numerical aperture, microlens *vs.* objective respectively). Ordinarily this loss in light gathering power would significantly decrease the signal to noise ratio for a typical single molecule experiment with organic fluorophores. There are two main contributors to noise in confocal microscopy: detector dark noise and autofluorescence. The first is constant and dominates at low light intensity, and the second is proportional to light intensity and dominates at high intensity. At very low signal intensities, a fundamental statistical noise component also arises equal to the square root of the number of photons counted. Under GeneEngine™ operating conditions, the signal to noise ratio is constant over excitation powers around those typically used, and therefore the detector dark noise has little impact on S/N ratio. However, a five-fold loss in light gathering power would overcome the GeneEngine™ S/N resolution. Compounding the issue of overall light collection efficiency are the affects of other non-ideal properties of microlenses. For instance, microlenses produce chromatic aberration (different focal lengths for different wavelengths) in which collection will always be somewhat out of focus in comparison to excitation. This effect could be as detrimental as the loss in light gathering power. However, the tremendous signal increase

available with quantum dots should more than compensate for the relatively poorer optical quality of microlenses in comparison to objectives.

Microlenses can be injection molded from high index plastics like Zeonor™ adding a negligible cost to each chip and eliminating a significant cost to the system. Microlenses span sizes from 300 μm or smaller to as large as 1.5 mm. Therefore a tight packed surface array of a hundred spherical microlenses is accomplished on a chip not much larger than a square centimeter, although with low cost plastics much larger chips with higher extents of parallelization are contemplated. A secondary advantage of the tight packed lens array is that broad collimated excitation light from a low cost lamp source can be used with minimal light losses occurring between the lenses. This configuration eliminates the high cost, blue laser light source otherwise required. Finally, microlenses are always in focus. Use of objectives requires expensive autonomous x-y-z micropositioning stages plus delays and extra complication for fine focusing and immersion oil dispensing.

As an alternative to microlens arrays, holographic optics can be used to generate an array of hundreds of diffraction limited confocal spots within the imageable area of an objective. Chip positioning under an objective can be accomplished for example with the alpha and beta version GeneEngine™ instruments which already contain this functionality. If necessary, a robust automated solution to micropositioning can also be used. The much higher signal of quantum dots can ameliorate the elevated system costs of both objectives and micropositioning effects. For example, less sophisticated objectives and lower magnification could be used increasing the microscope depth of field to ease the tolerances required for fine focusing.

A single electron multiplying CCD (EM-CCD) can be used to detect fluorescence emission from each confocal spot. In the traditional confocal setup, discrete avalanche photodiodes (APD) or photomultipliers (PMT) are most often used. The GeneEngine™ typically uses three discrete APDs to detect fluorescence in different colors and locations. Each APD costs approximately $2000, therefore parallelization compatible with 100 microlenses requires an inherently different approach. EM-CCDs are an emerging technology with on-chip signal amplification that essentially eliminates the largest source of noise in conventional CCDs, the read-out noise. (See, for example, Andor Technology website.) An EM-CCD based GeneEngine™ has been developed for single confocal spot detection using a first generation Andor iXon EM-CCD camera. This technology apparently performs

comparably to APDs. Furthermore, the bulk pricing on a custom EM-CCD camera is comparable to the cost of our current APDs.

Signal readout from each spot can be performed at the same 10 kHz rate used currently, and readout drive electronics can be customized for complex and rapid region-of-interest (ROI) selection provided certain limitations are met. Specifically, whole image rows can be passed over in selection at the readout serial clock rate, but columns must be read out one-by-one at the same clock rate. In other words, the typical readout design allows rapid access of rows containing the ROI, but the row itself must be read pixel-by-pixel until the end of the ROI is reached. Any blank space preceding the ROI, or between ROI elements, represents wasted time. This readout strategy is common to most CCDs, but does not represent a fundamental limitation. Additional acceleration of the serial clock between ROI elements in a row may be possible. Moreover, the optical coupling of fluorescence emission to the detector if chosen carefully may override this limit. One such possibility is to cluster the detection spots together in the readout corner of the device such that time accessing the first row and first column is minimized. With such a strategy, a fairly large area comprising a few hundred pixels can be read out at typical 10 kHz rates. Slower readout is certainly possible and would allow larger ROIs for higher parallelization.

The same general strategy of the standard GeneEngine™ configuration for spectral separation is envisioned here as a generalized two dimensional array. One strategy for minimizing false positives requires fluorescence monitoring in four different colors. In one embodiment, each bound agent complex contains two quantum dots emitting at separate wavelengths (e.g., green and red). Statistical correlation between for example red and green fluorescence signals indicates agent exposure. The statistical correlation in the spectral regions immediately adjacent to the tag emission establishes the background.

Devices are commercially available that split up images based on color using standard dichroic mirrors and narrow band pass filters. (See for example Optical Insights, LLC website.) The images in different colors are displaced with respect to each other into four quadrants of the array. Agent presence (and thus exposure) is assessed by correlating signals between the four quadrants on the array detector.

Agent exposure is detected by statistical correlation between the fluctuations in fluorescence intensity from the two differently colored tags. The goal of signal processing is to uncover the small correlated signal associated with the two-colored complexes amidst the gross excess of randomly correlated signals from the free fluorophores. Signal cross-

correlation is the ideal statistical approach for this task because only coincidence of fluorescence in time and space contributes to the cross-correlation. Random coincidence averages out. This single molecule detection approach has been demonstrated for mRNA quantitation with dual colored separate nucleic acid oligomer probes. (Korn, K. *et al.* (2003) Gene Expression Analysis Using Single Molecule Detection. *Nucleic Acids Research* 31(16): e89.) In this case, only the presence of the target mRNA brings the two separately colored fluorophores together. Similarly, in our case only the presence of an agent can lead to a correlated signal between for example two quantum dots of different colors. In general, dual color correlation is favored when samples have high free tag to target ratios. *In silico* studies were performed to assess the limits of dual color correlation to detect and/or quantify targets under these conditions. GeneEngine™ data was simulated at 1000:1 free tag to target ratio with the concentrations specified above, and the model was allowed to run until a statistically significant signal appeared above the random correlation. It was determined, on average, that 44 minute GeneEngine™ runs were required to establish exposure. Therefore, with 100× parallelization, bound and separated agents can be detected in under 30 seconds.

Detection time depends on agent concentration. At 10 fM, it typically takes the GeneEngine™ 1-5 minutes to make a positive identification, consistent with the volume of fluid passing through the confocal spot during that time. Assuming 100× concentration, it would take about ten times longer to identify a pathogen, but much less time for a toxin. Accelerating identification requires either increasing the flow rate, or increasing the cross-sectional interrogation area perpendicular to flow. Additionally, multiple detection channels could be added, and the concentration could be increased towards 1000×.

Secondary affinity partners can be derivatized in a number of ways. As discussed above, in some instances, the preferred derivatization includes an enzyme/substrate system such as the enzymatic hydrolysis of fluorescein diphosphate by alkaline phosphatase, or a direct binding to quantum dots. Coincidence of two or more colors is a powerful yet simple statistical tool for minimizing false positives. The likelihood is small that quantum dots of different colors would arrive at the detector at the same time by random chance after washing. Therefore, it is reasonable to use simultaneous detection of quantum dots of different colors to increase the confidence level of true agent identification.

The invention contemplates various detection modes, including direct labeling with quantum dots and indirect labeling and detection with enzymes. Examples of indirect enzyme-substrate systems include horseradish peroxidase, alkaline phosphatase, beta-

galactosidase, glucoamylase, lysozyme, luciferases such as firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456); saccharide oxidases such as glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase; heterocyclic oxidases such as uricase and xanthine oxidase coupled to an enzyme that uses hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase), and the like. The ultimate product is detected by its ability to emit and/or absorb electromagnetic radiation of a particular wavelength.

In other instances, a direct detection system is contemplated. Directly detectable labels include a chromogenic molecule, a fluorescent molecule (e.g., fluorescein isothiocyanate (FITC), TRITC, rhodamine, tetramethylrhodamine, R-phycoerythrin, Cy-3, Cy-5, Cy-7, Texas Red, Phar-Red and allophycocyanin (APC)), a chemiluminescent molecule, a bioluminescent molecule, an electroluminescent molecule, an optical or electron density molecule, an electromagnetic molecule, an electrical charge transducing or transferring molecule, a semiconductor nanocrystal or nanoparticle, an electron spin resonance molecule (such as for example nitroxyl radicals), a colloidal metal, a colloid gold nanocrystal, or a microbead. The label may be of a chemical, lipid, carbohydrate, peptide or nucleic acid nature although it is not so limited. Those of ordinary skill in the art will know of other suitable labels for the binding assay components, or will be able to ascertain such information using routine experimentation.

The label may be conjugated for example to a secondary affinity partner or to a solid support. As used herein, "conjugated" means two entities stably bound to one another by any physiochemical means. It is important that the nature of the attachment is such that it does not substantially impair the effectiveness of either entity. Keeping these parameters in mind, any covalent or non-covalent linkage known to those of ordinary skill in the art may be employed. In some embodiments, covalent linkage is preferred. Noncovalent conjugation includes hydrophobic interactions, ionic interactions, high affinity interactions such as biotin-avidin and biotin-streptavidin complexation and other affinity interactions. Such means and methods of attachment are known to those of ordinary skill in the art. Furthermore, the coupling or conjugation of these labels to the binding assay components of the invention can be performed using standard techniques common to those of ordinary skill in the art. For example, U.S. Patent Nos. 3,940,475 and 3,645,090 demonstrate conjugation of fluorophores and enzymes to antibodies.

Detectors can be selected from any number known in the art, and obviously corresponding to the labeling system used. In preferred embodiments, the detector is a fluorescent detector. In some important instances, the detector is an optical detector, confocal laser microscopy detector, a photographic film detector, or a chemiluminescent detector. In other instances, the detector is a charge coupled device (CCD) detector, an electron spin resonance (ESR) detector, an electrical detector, an electron microscopy detector, an atomic force microscopy (AFM) detector, a scanning tunneling microscopy (STM) detector, a scanning electron microscopy detector, an electron density detector, a refractive index detector, a near field detector, or a total internal reflection (TIR) detector.

The invention is premised in part on the ability to recycle key reagents, thereby reducing cost. The strategy for recycling will depend upon the nature of the solid support. Magnetic separation of reagents from sample however is common to the recycling process. If the solid support is itself a magnetic bead or particle, then the recycling and washing away of sample is more straightforward than if the solid support is a quantum dot. It is to be understood that recycling should preferably only be applied when a prior sample does not contain an agent.

Recycling steps are implemented between consecutive detection and binding steps. In the instance where the solid support is a magnetic bead or particle, a magnetic force is applied to the chamber, thereby allowing the movement and maintenance of beads with affinity partners affixed thereto in the chamber. The environment within the chamber should be manipulated in order to remove all sample remnants from the chamber. This may include increasing temperature, changing pH, varying salt concentration and the like. Such manipulation must remove all sample traces while not adversely affecting the affinity partners which are to be recycled repeatedly. Once this is complete, the magnetic force may be removed, thereby allowing the beads or particles to enter the chamber buffer and contact agent in the next sample to be tested.

In the case of quantum dot recycling, the quantum dots may be derivatized with one or more oligomers. Once the detection step is completed, magnetic beads or particles that are themselves derivatized with the complement of the quantum dot oligomer are allowed to contact and bind to the quantum dots (via complementarity of the respective oligomers). The magnetic beads or particles are allowed to contact the quantum dots, for example, by removing a magnetic force that was present during the binding and detection steps. Once the complementary single stranded oligomers have hybridized to each other, the magnetic force is

re-applied and the chamber is manipulated as before to wash the chamber free of sample. Once the chamber is washed sufficiently, it is further manipulated to dissociate the oligomers from each other. It is this step that controls the length of the oligomer to be used (and vice versa): the longer the oligomer length, the more severe and prolonged will be the conditions necessary to dissociate the hybrid. Accordingly, shorter oligomers are preferred provided they give rise to sufficiently stable hybrids during the wash period.

The oligomer can be of any size, however, preferably it is less than 100 bases, more preferably less than 50 bases, and even more preferably less than 20 bases (e.g., 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, or fewer nucleotides). In one embodiment, the oligomer is at least 13 residues in length.

The oligomer will preferably be a nucleic acid polymer such as a DNA or RNA oligomer. It may also be comprised of PNA or LNA elements, or combinations thereof. The oligomer may further comprise one or more backbone modifications that render it less susceptible to enzymatic degradation. Such backbone modifications are known in the art and include but are not limited to phosphorothioate modifications, methylphosphonate modifications, methylphosphorothioate modifications, phosphorodithioate modifications, p-ethoxy modifications, and combinations thereof. This list is not intended to be exhaustive.

The invention contemplates hybrid dissociation using temperature primarily, although salt concentration, pH and other parameters may also be used. The melting point of complementary nucleic acid oligomers is size-dependent. Most hybrids (particularly those of the size contemplated by the invention) will dissociate at about 40 °C. For example, a 13-mer melts from its complement at a temperature that is safe for antibodies, yet high enough for efficient reagent recovery. IgG and IgY antibodies can be quite thermostable (~75% functional after 80 days at 40 °C for rabbit IgG, goat IgG and duck IgY) (Chiou, V. (2002) Duck Antibodies for IVD Applications, Device Link website), recombinant single chain antibodies are similarly durable (100% functional after 7 days at 37 °C) (Maynard, J. et al. (2002) Protection Against Anthrax Toxin by Recombinant Antibody Fragments Correlates with Antigen Affinity. *Nature Biotechnology* 20:597-601), and camelid antibodies are even more resistant to heat-denaturation with apparent melting temperatures between 60-80 °C (Demoulin et al. (2002) Single-Domain Antibody Fragments with High Conformational Stability. *Protein Science*, 11:500-515).

As an alternative to oligomers, the quantum dot may be derivatized with protein A as a way of binding affinity partners that are antibodies. Protein A is commonly used in antibody

purification since it binds strongly to subclasses of IgG antibody, and releases upon acidification. Protein A complexes would thus require changes in pH in order to effect dissociation, either through titration or redox reaction.

The magnetic force may derive from a moveable rare earth magnet capable of sequestering the beads away from the convective flow that carries away the sample and reagents. After detection, the flow is stopped and the magnet is removed allowing interaction of beads and the derivatized and potentially bound quantum dots. To ensure efficient reagent recovery, extra magnets or other forms of mechanical mixing can be employed if necessary. After detection, the reagent-bead complexes are magnetically isolated from the convective flow for washing and heat-induced dissociation, as described above.

Magnetic separation and washing of magnetic particles or beads can be comparatively fast with proper design of the mixing chamber. It is reasonable to expect that the reaction volume could be reduced 100-fold, bringing the biological or chemical agent concentrations up to detectable femptomolar levels. It is possible that free magnetic particles or beads (i.e., those that do not have a biological or chemical agent bound thereto) outnumber bound complexes by about 10^{10}-to-1, and therefore another biochemical reaction may be needed to overcome such background.

The reagents may be recycled once, twice, thrice, four times, or five times, but preferably are recycled at least 10 times, at least 20 times, at least 50 times, at least 100 times, at least 200 times, at least 500 times, or more.

Maintaining a sufficient reagent concentration will be important for effective recycling. The reagent concentration drops by the reagent collection efficiency raised to the power of the number of reuses. For example, at a hypothetical 80% collection efficiency only 10% of the reactant would remain after ten re-uses. To maximize reagent recovery, the capture reaction will be allowed to equilibrate with a high excess of complementary oligomers, in the quantum dot embodiment. Preliminary experimental results indicate this should take about five minutes to complete, plus a few more minutes for washing and resuspension. Microchips may be taken off-line during regeneration. Chip use may also be cycled such that each chip has sufficient time for regeneration between uses. It is expected that about ten chips will be in use at any one time, as an example.

An example of one system embodiment includes a milliliter volume sealable well that may be positioned on the backside of a chip device (with lenses on the front side) such that the microfluidic loading port is in fluidic contact even before centrifugation. The crude

filtrate from the aerosol collector is deposited directly into this well. The chip itself is spun such that cells, spores, viruses and the like sediment towards the loading port. After loading a microliter of sample into the chip, acoustical conditioning of the entire chip will disrupt the pathogens. Fine filtration may be accomplished on-chip by a post field array with post spaced for example at 50 micron intervals. The filter can be regenerated by flushing in reverse. Still inside the microfluidic environment, the filtered sample is delivered by convective flow to a reaction chamber where heat-induced reactant release from the magnetic microspheres forms the reaction complexes. The sample is driven directly from the reaction chamber through the HDC column to the microlens array end of the column for detection. The column empties into a second reaction chamber with more magnetic beads for device regeneration. In an efficient symmetrical chip design, the reactants will bounce back and forth between two regeneration/reaction chambers, with HDC and detection in between.

Equivalents

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the invention. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

What is claimed is:

Claims

1. A method for detecting a biohazardous agent comprising

contacting a sample to a recycled first affinity partner conjugated to a first detectable solid support,

contacting the sample to a recycled second affinity partner conjugated to a second detectable solid support, and

determining binding of the first and second affinity partner to a biohazardous agent based on two presence of two color coincidence,

wherein two color coincidence indicates the presence of the biohazardous agent.

2. The method of claim 1, wherein the recycled first affinity partner is present at a concentration of about 100 nM.

3. The method of claim 1, wherein the recycled second affinity partner is present at a concentration of about 100 nM.

4. The method of claim 1, wherein the first affinity partner is an antibody, an antibody fragment, or an aptamer.

5. The method of claim 1 or 4, wherein the second affinity partner is an antibody, an antibody fragment, or an aptamer.

6. The method of claim 1, wherein the first detectable solid support is a quantum dot.

7. The method of claim 1, wherein the second detectable solid support is a quantum dot.

8. The method of claim 6 or 7, wherein the quantum dot is conjugated to an oligomer.

9. The method of claim 1, wherein the first detectable solid support and the second detectable solid support is fluorescently detectable.

10. The method of claim 1, wherein the sample is filtered.

11. The method of claim 1, wherein the sample contains disrupted pathogens.

12. The method of claim 1, wherein the sample has been concentrated by centrifugation.

13. The method of claim 1, wherein the sample is volume condensed.

14. The method of claim 1, wherein the sample is derived from an air sample, a liquid sample, or a swab or swipe sample.

15. The method of claim 1, wherein the sample is present in a solvent.

16. The method of claim 15, wherein the solvent is an aqueous solvent.

17. The method of claim 1, wherein the first and second recycled affinity partners are recycled by application of a magnetic field.

18. The method of claim 1, wherein the biohazardous agent is a biological agent.

19. The method of claim 1, wherein the biohazardous agent is a weaponized agent.

20. The method of claim 19, wherein the biological agent is a bacterium, a bacterial spore, a virus, a fungus, a parasite, a mycobacterium, or a mycoplasma.

21. The method of claim 19, wherein the biological agent is a prion.

22. The method of claim 19, wherein the biological agent is a toxin.

23. The method of claim 1, further comprising separation of first and second detectable solid supports that are bound to an agent from first and second supports that are not bound to an agent.

24. The method of claim 23, wherein the separation is performed by chromatography.

25. The method of claim 1, wherein the two color coincidence is detected using a single molecule analysis system.

26. The method of claim 1, wherein the single molecule analysis system comprises a parallel detection system.

27. The method of claim 1, wherein the parallel detection system comprises a microlenses system.

28. A system for detecting a biohazardous agent comprising
a sample collection device,
a recycled first affinity partner conjugated to a first detectable solid support,
a recycled second affinity partner conjugated to a second detectable solid support,
a magnetic bead or particle derivatized with an third oligomer,
a magnetic separation device, and
a detection system,
wherein the first solid support is conjugated to a first oligomer and the second solid support is conjugated to a second oligomer, and wherein the first and second oligomers bind to individual third oligomers.

29. The system of claim 28, wherein the sample collection device is an air sample collection device.

30. The system of claim 28, wherein the recycled first affinity partner is present at a concentration of about 100 nM.

31. The system of claim 28, wherein the recycled second affinity partner is present at a concentration of about 100 nM.

32. The system of claim 28, wherein the first affinity partner is an antibody, an antibody fragment, or an aptamer.

33. The system of claim 28 or 32, wherein the second affinity partner is an antibody, an antibody fragment, or an aptamer.

34. The system of claim 28, wherein the recycled first affinity partner and second affinity partner bind to different epitopes on the same biohazardous agent.

35. The system of claim 28, wherein the recycled first affinity partner and second affinity partner bind to the same epitope on the same biohazardous agent

36. The system of claim 28, wherein the first solid support is fluorescently detectable.

37. The system of claim 28 or 36, wherein the second solid support is fluorescently detectable.

38. The system of claim 28, wherein the first and second solid support is a quantum dot.

39. The system of claim 28, further comprising a sample condensing device.

40. The system of claim 28, wherein the sample collection device is the sample condensing device.

41. The system of claim 28, wherein the system comprises microfluidic chip.

42. The system of claim 28, wherein the biohazardous agent is a biological agent.

43. The system of claim 42, wherein the biohazardous agent is a weaponized agent.

44. The system of claim 42, wherein the biological agent is a bacterium, a bacterial spore, a virus, a fungus, a parasite, a mycobacterium or a mycoplasma.

45. The system of claim 42, wherein the biological agent is a prion.

46. The system of claim 42, wherein the biological agent is a toxin.

47. The system of claim 28, wherein the detection system is a single molecule detection system.

48. A method for detecting a biohazardous agent comprising

contacting a sample to a recycled first affinity partner conjugated to a magnetic support,

isolating the magnetic support, and

contacting the magnetic support with a second affinity partner that is detectable, and

determining binding of the second affinity partner to the magnetic support,

wherein binding of the second/secondary affinity partner indicates the presence of a biohazardous agent.

49. The method of claim 48, wherein the recycled first affinity partner is present at a concentration of about 100 nM.

50. The method of claim 48, wherein the first affinity partner is an antibody, an antibody fragment, or an aptamer.

51. The method of claim 48 or 50, wherein the second affinity partner is an antibody, an antibody fragment, or an aptamer.

52. The method of claim 48, wherein the sample is filtered.

53. The method of claim 48, wherein the sample is volume condensed.

54. The method of claim 48, wherein the sample is derived from an air sample, a liquid sample, or a swab or swipe sample.

55. The method of claim 48, wherein the sample is present in a solvent.

56. The method of claim 55, wherein the solvent is an aqueous solvent.

57. The method of claim 48, wherein the magnetic support is a magnetic bead or a magnetic particle.

58. The method of claim 48, wherein the magnetic support is isolated by application of a magnetic field.

59. The method of claim 1 or 48, wherein the biohazardous agent is a biological agent.

60. The method of claim 48, wherein the biohazardous agent is a weaponized agent.

61. The method of claim 59, wherein the biological agent is a bacterium, a bacterial spore, a virus, a fungus, a parasite, a mycobacterium, or a mycoplasma.

62. The method of claim 59, wherein the biological agent is a prion.

63. The method of claim 59, wherein the biological agent is a toxin.

64. The method of claim 48, wherein the second affinity partner is fluorescently detectable.

65. A system for detecting a biohazardous agent comprising
a sample collection device,

a recycled first affinity partner conjugated to a magnetic support,
a magnetic separation device,
a second affinity partner that is detectable, and
a detection system.

66. The system of claim 65, wherein the sample collection device is an air sample collection device.

67. The system of claim 65, wherein the recycled first affinity partner is present at a concentration of about 100 nM.

68. The system of claim 65, wherein the first affinity partner is an antibody, an antibody fragment, or an aptamer.

69. The system of claim 65 or 68, wherein the second affinity partner is an antibody, an antibody fragment, or an aptamer.

70. The system of claim 65, wherein the recycled first affinity partner and second affinity partner bind to different epitopes on the same biohazardous agent.

71. The system of claim 65, wherein the recycled first affinity partner and second affinity partner bind to same epitopes on the same biohazardous agent.

72. The system of claim 65, wherein the second affinity partner is fluorescently detectable.

73. The system of claim 65, further comprising a sample condensing device.

74. The system of claim 73, wherein the sample collection device is the sample condensing device.

75. The system of claim 65, wherein the system comprises microfluidic chip.

76. The system of claim 65, wherein the magnetic support is a magnetic bead or a magnetic particle.

77. The system of claim 65, wherein the biohazardous agent is a biological agent.

78. The system of claim 77, wherein the biohazardous agent is a weaponized agent.

79. The system of claim 77, wherein the biological agent is a bacterium, a bacterial spore, a virus, a fungus, a parasite, a mycobacterium, or a mycoplasma.

80. The system of claim 77, wherein the biological agent is a prion.

81. The system of claim 77, wherein the biological agent is a toxin.

82. The system of claim 65, wherein the detection system is a single molecule detection system.

83. The system of claim 65, wherein the magnetic separation device is a magnetic field.

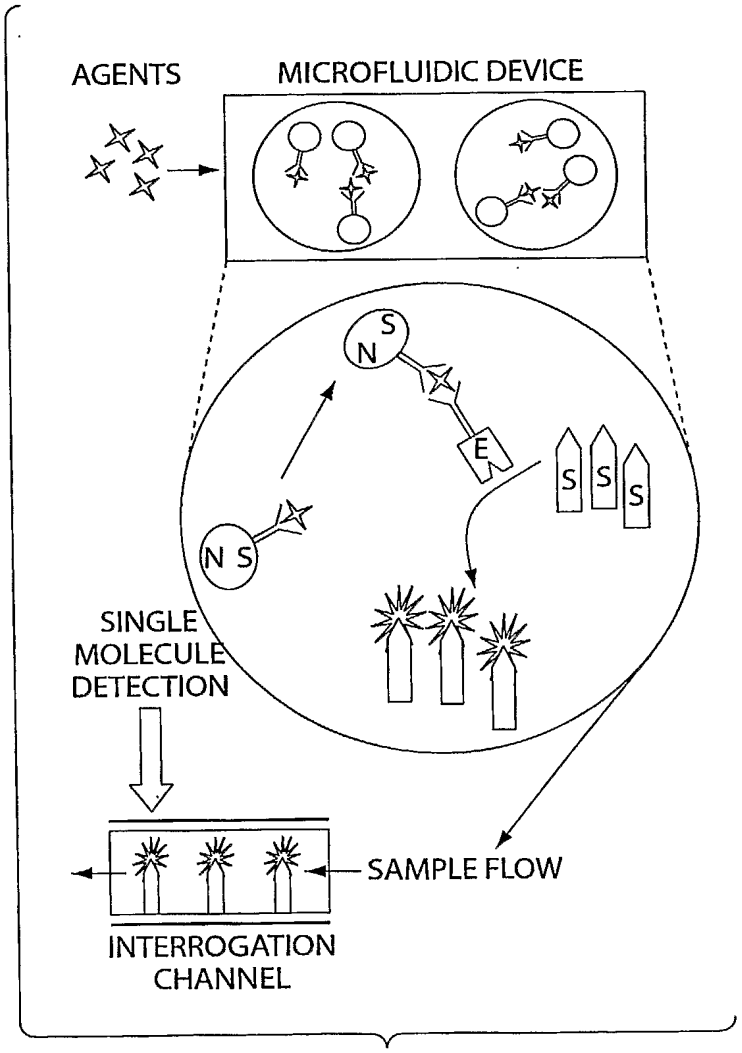
AGENTS
MICROFLUIDIC DEVICE
N
S
E
S
S
S
N S
SINGLE
MOLECULE
DETECTION
SAMPLE FLOW
INTERROGATION
CHANNEL

Fig. 1

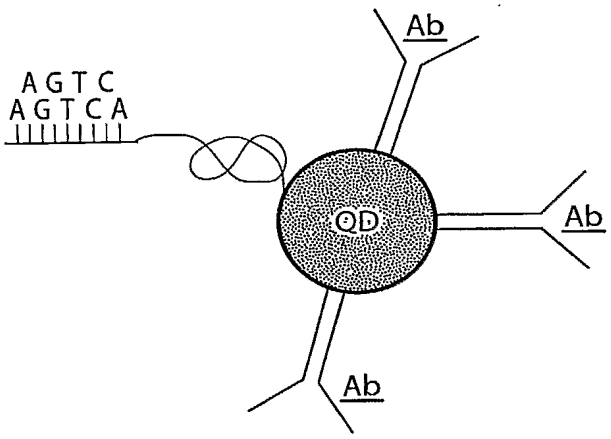

Fig. 2

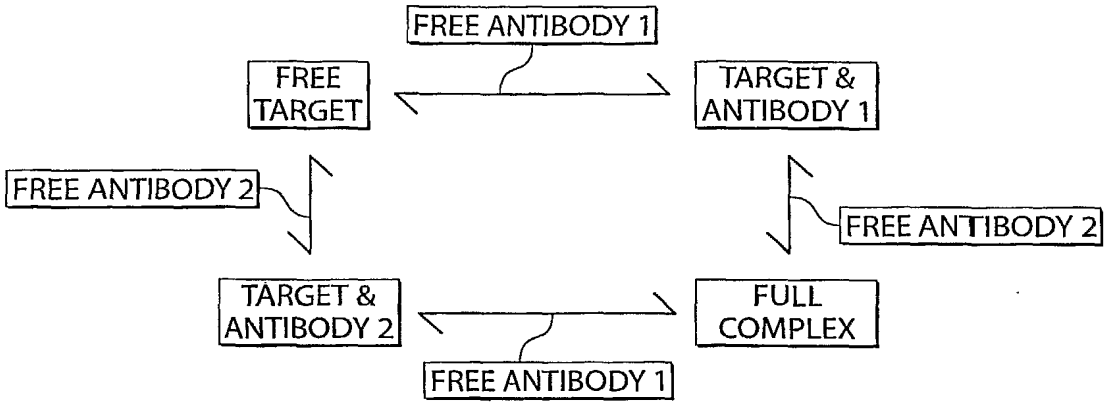

Fig. 3A

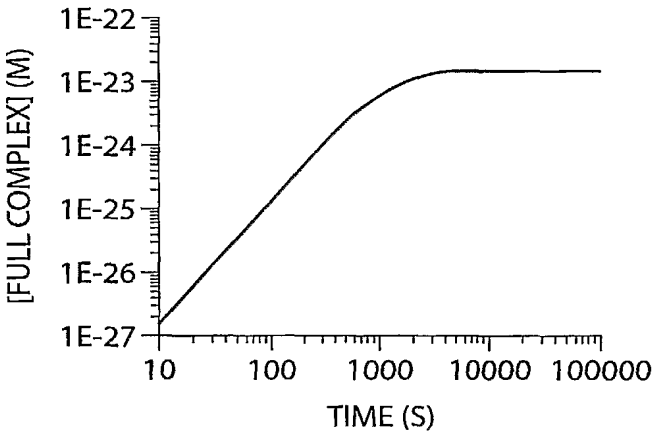

Fig. 3B

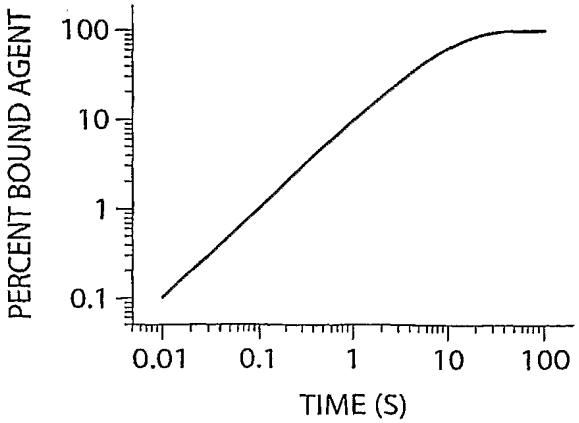

Fig. 3C

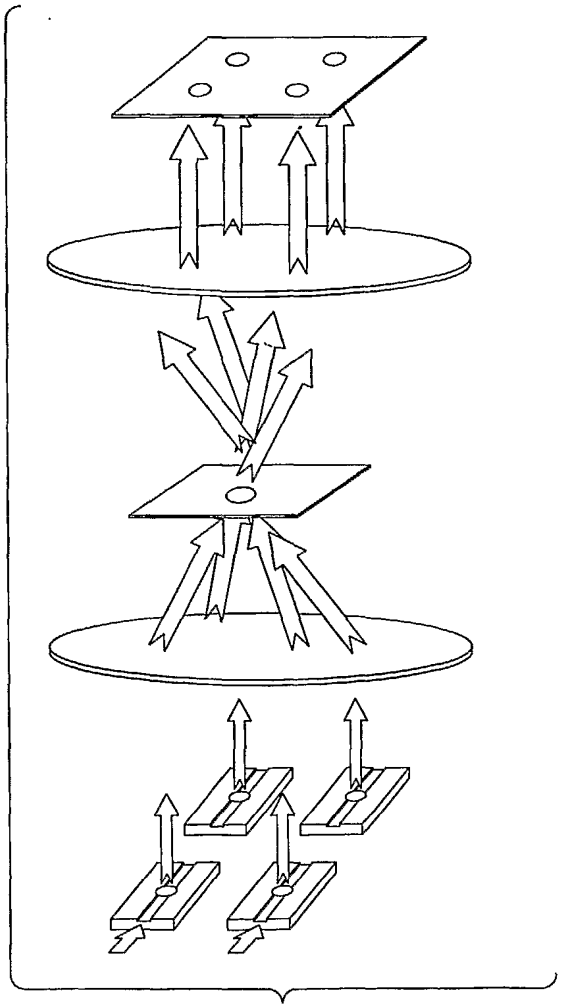

Fig. 4